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For:	**Use of Malate Dehydrogenase for NADH Regeneration**
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Use of Malate Dehydrogenase for NADH Regeneration

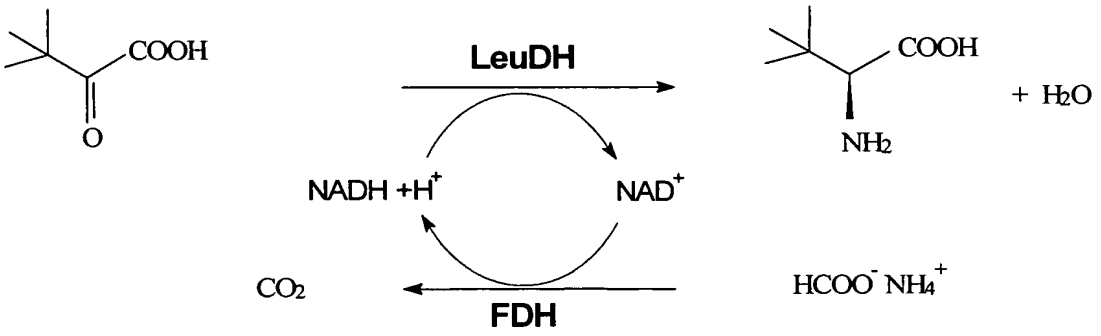
Field of the Invention

The present invention relates to a process for the preparation of enantiomerically enriched organic compounds. In particular, the invention relates to a coupled enzymatic reaction system, in which NAD(P)H is consumed by one enzyme for the preparation of the organic compound and NAD(P)H is simultaneously regenerated by a second enzyme system. The invention also includes a reaction system which operates in this manner and whole cell catalysts or plasmids.

Background of the Invention

The production of optically active organic compounds, by a biocatalytic route is increasingly gaining importance. The coupled use of two dehydrogenases with cofactor regeneration has emerged, inter alia, as a route for the large-scale industrial synthesis of these compounds, in particular alcohols and amino acids (DE19753350, EP118750).

Equation 1:



Equation 1 shows the *in situ* regeneration of NADH with the NAD-dependent formate dehydrogenase from *Candida boidinii* in the reductive amination of trimethyl pyruvate to give L-tert-leucine (Bommarius *et al.*, *Tetrahedron Asymmetry* 6:2851-2888(1995)).

In addition to their catalytic properties and efficiency, biocatalysts have the advantage that, in contrast to a large number of synthetic metal-containing catalysts, the use

of toxic metal-containing starting substances can be dispensed with. The use of expensive and hazardous reducing agents, *e.g.*, borane, can also be dispensed with.

The FDH, *e.g.*, from *Candida boidinii*, that has been successfully employed in these systems has the disadvantage that the specific activity of this enzyme class at 4-8 U/mg is very low. This necessitates the use of a large amount of expensive enzyme with recycling. As a result it is difficult to perform processes on an industrial scale economically.

Malate dehydrogenase (MDH), also called "malic enzyme," catalyses the oxidative decarboxylation of malate to pyruvate. Numerous malate dehydrogenases are known, including enzymes from higher animals, plants and microorganisms. A distinction is made between four types of malate dehydrogenases, which are classified into the enzyme classes E.C. 1.1.1.37 to E.C. 1.1.1.40 (http://www.genome.ad.jp). NAD and/or NADP is required as a cofactor, depending on the type of malate dehydrogenase used.

Due to the irreversibility of the oxidative decarboxylation reaction of L-malic acid to pyruvate, the use of malate dehydrogenase is also appropriate in the systems described. The use of malate dehydrogenase for regeneration of NAD is described, for example, by Suye, *et al.*, *Can. J. Chem. Eng. 70*:306-312 (1992)). NADH regeneration by means of malate dehydrogenase is used for reductive amination of pyruvate by means of an alanine dehydrogenase, NADH being consumed. The pyruvate is formed by oxidative decarboxylation from L-malic acid, and is immediately consumed again in the following step by alanine dehydrogenase. The problem of inhibition due to the presence of large amounts of pyruvate is therefore avoided. Nevertheless, the system is limited exclusively to the production of alanine (see Suye, *Recent Res. Devel. Ferment. Bioeng. 1*:55-64 (1998)).

Description of the Invention

The present invention is directed to a process for the preparation of chiral organic compounds, such as amino acids or alcohols, using a coupled malate dehydrogenase reaction system which is not limited to the preparation of one substance. It should be possible to use this process on an industrial scale in a manner that is both economically and ecologically advantageous.

More specifically, the invention is directed to a process for the preparation of enantiomerically enriched organic compounds in a coupled enzymatic reaction system. The system comprises a first enzymatic transformation of an organic substrate, during which NAD(P)H is consumed, and a second enzymatic transformation, during which the NAD(P)H is regenerated. The second enzymatic transformation is catalyzed by malate dehydrogenase and invoves the oxidation of L-malic acid to pyruvate and CO_2. The pyruvate formed from the second enzymatic transformation is not used as the substrate in the first enzymatic transformation. Surprisingly, the simultaneous use, *e.g.*, of an amino acid dehydrogenase with malate dehydrogenase, is possible without problems due to cross-reactivity. In addition, the substrates and products of the primary reaction do not appear to inhibit the malate dehydrogenase reaction or vice versa. In particular, it is a positive feature that the pyruvate formed as a by-product does not inhibit the malate dehydrogenase itself or the alcohol or amino acid dehydrogenases employed in parallel.

Enantiomerically enriched alcohols or amino acids can be prepared with the process of the present the invention. In this case, inexpensive alcohol dehydrogenase or amino acid dehydrogenase may be used as the enzyme for the first enzymatic transformation. Specific enzymes that may be used are described by Drauz, *et al.*, (Enzyme Catalysis in Organic Synthesis, volume III, Wiley-VCH, Weinheim, chapter 15, (2002)). The use of the alcohol dehydrogenase from either the organism *Rhodococcus erythropolis* (S-ADH) (Peters, *et al.*, *J. Biotechnol. 33*:283-292 (1994)) or *Lactobacillus kefir* (R-ADH) (Bradshaw, *et al.*, *J. Org. Chem. 57*:1532-1536 (1992)) is especially preferred. Preferred amino acid dehydrogenases, for example, leucine dehydrogenases or phenylalanine dehydrogenases, are described by Bommarius in: Enzyme Catalysis in Organic Synthesis (eds.: K. Drauz, H. Waldmann), volume III, Wiley-VCH, Weinheim, chapter 15.3, (2002).

Malate dehydrogenases are also well known in the art. In principle, those malate dehydrogenases which regenerate NAD(P)H to an extent such that a bottleneck does not arise during the course of the reaction of the other enzyme should be employed. The malate dehydrogenase from *E. coli* K12 is preferred in this connection.

In principle, the process according to the invention can be carried out in purely aqueous solution. However, it is also possible to add a water-soluble organic solvent to the

aqueous solution in order to, for example, optimize the reaction for poorly water-soluble substrates. Examples of such solvents include ethylene glycol, DME and glycerol. Multi-phase, in particular two-phase, systems comprising an aqueous phase can also serve as the solvent mixture for the process. The use of certain solvents which are not water-soluble has been described in DE10233107 and the statements made therein also apply to the present process.

One of skill in the art will choose the temperature present during the reaction, preferably with the objective of obtaining the highest possible yield of product, in the highest possible purity, in the shortest possible time. The enzymes employed should be stable under the temperature chosen and the reaction should proceed with the highest possible enantioselectivity. When using enzymes from thermophilic organisms, it is entirely possible for temperatures of 100°C to represent the upper limit of the temperature range in the reaction. A temperature of -15°C is appropriate as the lower limit in aqueous systems. In general, a temperature interval between 10 and 60, and preferably between 20 and 40°C will typically be used in reactions.

The pH during the reaction is determined based upon enzyme stability and conversion rates. For the malate dehydrogenase from *E. coli*, it has been found that the optimum pH is > 10. In general, the range preferred for enzymes will be from pH 5 to 11, more preferably from 5.5 to 10.0, and still more preferably from 6.0 to 9.0.

The reaction system is advantageously employed, for example, in a stirred tank, a cascade of stirred tanks or in membrane reactors, which can be operated both in batch operation and continuously. In the context of the invention, the term "membrane reactor" is understood as meaning any reaction vessel in which the catalyst is enclosed in a reactor, while low molecular weight substances are fed to the reactor or can leave it. The membrane can be integrated directly into the reaction space or incorporated outside in a separate filtration module, through which reaction solution flows continuously or intermittently and the retained product is recycled into the reactor. Suitable embodiments are described, *inter alia*, in WO98/22415; in Wandrey *et al.* (in Yearbook, Verfahrenstechnik und Chemieingenieurwesen, VDI p. 151 et seq. (1998); in Applied Homogeneous Catalysis with Organometallic Compounds, vol. 2, VCH, p. 832 et seq. (1996)); and in Kragl *et al.*,

(*Angew Chem. 6:*684 et seq (1996)). The continuous procedure which is possible in this apparatus, in addition to the batch and semi-continuous procedure, can be carried out in the cross-flow filtration mode (fig. 3) or as a dead-end filtration (fig. 2). Both process variants are described in the prior art (Engineering Processes for Bioseparations, L.R. Weatherley, 135-165 (1994); Wandrey *et al.*, *Tetrahedron Asymmetry 10*:923-928 (1999)).

The present invention also provides whole cell catalysts comprising a cloned gene for a first enzyme for transformation of an organic substrate and a cloned gene for a malate dehydrogenase. The first enzyme is capable of preparation of an enantiomerically enriched organic compound in a first enzymatic transformation, during which NAD(P)H is consumed. The NAD(P)H is regenerated in a second enzymatic transformation catalyzed by malate dehydrogenase, in which L-malic acid is oxidized to pyruvate and CO_2. The pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation. The whole cell catalyst according to the invention preferably has an enzyme (polypeptide) with amino acid or alcohol dehydrogenase activity and one with malate dehydrogenase activity, preferably originating from the organisms mentioned above.

Microorganisms which can be used include: yeasts, such as *Hansenula polymorpha*, *Pichia sp.* and *Saccharomyces cerevisiae*; prokaryotes, such as *E. coli* and *Bacillus subtilis*; and eukaryotes, such as mammalian cells and insect cells. *E. coli* strains are preferably used. The following are particularly preferred: *E. coli* XL1 Blue, NM 522, JM101, JM109, JM105, RR1, DH5α, TOP 10' or HB101. An organism as mentioned in DE10155928 is preferably employed as the host organism. The advantage of such an organism is the simultaneous expression of both polypeptide systems, which means that only one organism has to be cultured for the reaction according to the invention.

To coordinate the expression of the polypeptides, the corresponding coding nucleic acid sequences can be accommodated on different plasmids with different numbers of copies and/or promoters of different potency. Ideally in such coordinated enzyme systems, no accumulation of an intermediate compound occurs, and the reaction in question can proceed at an optimum overall rate. These prnciples are well known in the art (Gellissen, *et al., Appl. Microbiol. Biotechnol. 4*646-54 (1996); DE19920712).

It is preferred that the whole cell catalyst according to the invention further metabolizes the pyruvate formed in the reaction and uses it as a nutrient source. Whole cell catalysts designed in this manner have the advantage that pyruvate is not obtained as a by-product of the reaction and therefore does not have to be separated off from the chiral product actually desired in further process steps.

The preparation of the whole cell catalyst can be carried out using methods that are well known in the art (Sambrook, *et al.*, Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989); Balbas, *et al.*, *Meth. Enzymol. 185*:14-37 (1990); Rodriguez, *et al.* (eds), Vectors: a survey of molecular cloning vectors and their uses, 205-225, Butterworth, Stoneham, 1988)). With respect to general methodology of molecular biology (PCR, cloning, expression etc.) reference may also be made to the following literature: Universal GenomeWalker™ Kit User Manual, Clontech, 3/2000 and literature cited therein; Triglia, *et al., Nucl. Ac. Res. 16:*8186 (1988); Sambrook, *et al.*, Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York 1989); and Rodriguez, *et al.* (eds), Vectors: a survey of molecular cloning vectors and their uses, Butterworth, Stoneham (1988)).

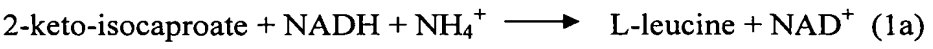
The invention also provides plasmids containing gene constructs in which a gene coding for a malate dehydrogenase and a gene coding for an enzyme which catalyzes the transformation of an organic substrate in a reaction accompanied by the consumption of NAD(P)H are present. Possible plasmids or vectors are well known in the art and can be found, *e.g.*, in Studier, *et al., Meth. Enzymol. 185*:61-89 (1990)) or the brochures of Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. Further preferred plasmids and vectors can be found in: Glover, D. M., DNA cloning: A Practical Approach, vol. I-III, IRL Press Ltd., Oxford (1985); Rodriguez, *et al.* (eds), Vectors: a survey of molecular cloning vectors and their uses, 179-204, (1988); Butterworth, *et al.*, *Meth. Enzymol. 185*:3-7 (1990); Sambrook, *et al.*, Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989).

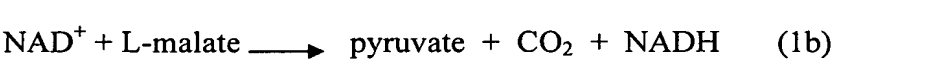
Preferred plasmids for cloning gene constructs include: pUC18 (Roche Biochemicals), pKK-177-3H (Roche Biochemicals), pBTac2 (Roche Biochemicals), pKK223-3 (Amersham Pharmacia Biotech), pKK-233-3 (Stratagene) ~~or~~ and pET

(Novagen). The plasmid pkk/phe/mali (fig. 5) may be used in this connection. The preparation of this plasmid and of a corresponding microorganism is described in the dissertation by S. Naamnieh (University of Düsseldorf, in preparation p. 70 et seq).

The polypeptides of the process according to the invention can be used in free form as homogeneously purified compounds or as an enzyme prepared by a recombinant method. These polypeptides can also be employed as a constituent of an intact guest organism or in combination with the broken-down cell mass of the host organism, which has been purified to any desired extent. The use of the enzymes in immobilized form is also possible (Sharma, *et al.*, *Angew Chem. 94*:836-852 (1982)). The immobilization is advantageously carried out by lyophilization (Paradkar, *et al., J. Am Chem. Soc. 116*:5009-5010 (1994); Mori, *et al., Tetrahedron Lett. 38*:1971-1974 (1997); Otamiri, *et al., Biocatalysis 6*:291-305 (1992)). Lyophilization in the presence of surface-active substances, such as Aerosol OT or polyvinylpyrrolidone or polyethylene glycol (PEG) or Brij 52 (diethylene glycol monocetyl ether) (Kamiya, *et al., Biotechnol. Tech. 11*:375-378 (1997)), is very particularly preferred. Immobilization on Eupergit®, in particular Eupergit C® and Eupergit 250L® (Röhm) (Katchalski-Katzir, *et al., J. Mol. Catal. B: Enzymatic 10:*157-176 (2000)), is still more preferred. Immobilization on Ni-NTA in combination with the polypeptide supplemented with the His tag (hexa-histidine) is likewise preferred Bornhorst, *et al., Meth. Enzymol. 326*:245-254 (2000)). The use as CLECs is also possible (St. Clair, *et al., Angew Chem. Int. Ed. 39*:380-383 (2000)). By these measures, it is possible to generate polypeptides which can operate in mixtures of aqueous and organic solvents or entirely in organic media.

The process according to the invention can be carried out such that the MDH from *E. coli* is coupled with an NAD-dependent leucine dehydrogenase (LeuDH from *Bacillus cereus*; Sigma). LeuDH catalyses the reductive amination of aliphatic keto acids, such as, for example, ketoisocaproate, to the corresponding L-amino acids, such as L-leucine, with consumption of NADH (equation (1)).





The course of the reaction is monitored by HPLC and results are shown in table 1 along with results from an equivalent conversion that is carried out with FDH instead of MDH.

Table 1: Comparison of the formation of L-leucine (HPLC) in a coupled batch with coenzyme regeneration by MDH and FDH. In each case, 10 mM ketoisocaproate is used. 100 mM L-malate is used for regeneration by malate dehydrogenase (MDH) and 100 mM formate is used for regeneration by formate dehydrogenase (FDH).

Time [min]	L-Leucine with MDH [mM]	L-Leucine with FDH [mM]
0	0.7	0.1
10	8.8	9.9
30	9.6	11.1
60	10.8	9.8
120	9.9	9.9

The use of alcohol dehydrogenases in combination with malate dehydrogenases is also possible. The MDH from *E. coli* is coupled with an NAD-dependent S-specific alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH; DE10218689). The usability of MDH is tested here via the reduction of a ketone (p-Cl-acetophenone = pCAp) in accordance with equation (4).

$$pCAp + NADH + H^+ \longrightarrow p\text{-Cl-phenylethanol} + NAD^+ \quad (4a)$$

$$NAD^+ + \text{L-malate} \longrightarrow \text{pyruvate} + CO_2 + NADH \quad (4b)$$

Table 2: Decrease in the ketone p-Cl-acetophenone (10 mM) and increase in the enzymatically formed alcohol p-Cl-phenylethanol (100%, corresp. to 10 mM) as a function of time.

Time [min]	Ketone [%]	Alcohol [%]
0	100	0
10	28	72
20	12	88
30	4	96
60	0	100

The term "enriched" or "enantiomer-enriched" describes the fact that one optical antipode is present in a mixture at >50%. If one stereo-centre is present, the structures shown relate to the two possible enantiomers, and if more than one stereo-centre is present in the molecule, they relate to all the possible diastereomers and, in respect of a diastereomer, to the two possible enantiomers of the compound in question.

The organism *Candida boidinii* is deposited under number ATCC 32195 at the American Type Culture Collection and is accessible to the public.

The documents of the prior art mentioned in this specification are hereby incorporated by reference herein.

Brief Description of the Drawings

Figure 1: Effect of pH on MDH Activity. MDH was isolated from *E. coli* and its activity at different pH values examined. Maximum activity occurred at pH values of 11 and higher. Nevertheless, good activity is still observed at pH values as low as 7.0. The experiment shown in the figure is more fully described in Example 1.

Figure 2: Membrane Reactor with Dead-End Filtration. Substrate (1) is transferred via a pump (2) into the reactor space (3), which contains a membrane (5). In the reactor space, which is operated with a stirrer, are, in addition to the solvent, the catalyst (4), the product (6) and unreacted substrate (1). Low molecular weight product (6) is chiefly filtered off via the membrane (5).

Figure 3: Membrane Reactor with Cross-Flow Filtration. The substrate (7) is transferred by pump (8) into the stirred reactor space, in which solvent, catalyst (9) and product (14) are also present. A solvent flow which leads via a heat exchanger (12), which may be present, into the cross-flow filtration cell (15) is established via the pump (16). The low molecular weight product (14) is separated off here via the membrane (13). High molecular weight catalyst (9) is then passed back with the solvent flow, if appropriate via a heat exchanger (12) again, if appropriate via the valve (11), into the reactor (10).

Figure 4: Effect of Temperature on MDH Activity: The figure shows the results of an experiment examining the effect of temperature on the activity of MDH isolated from *E. coli*. The optimum temperature of the MDH reaction is approximately 55°C.

Figure 5: The Plasmid pkk/phe/mali: The figure is a diagram of an expression vector encoding both phenylalanine dehydrogenase and malate dehydrogenase.

Figure 6: HPLC Analysis of Reaction Product: A whole cell conversion reaction was carried out (see Example 8). The phenylalanine formed in the reaction was assayed by HPLC and results are shown in the figure.

Figure 7: Product Yield: The phenylalanine formed in a whole cell conversion reaction (see Example 8) was monitored over a period of 20 hours and results are shown in the figure.

Figure 8: Construction of Plasmid containing pheDH and Malate Dehydrogenase: The figure shows diagrammatically the steps used in constructing a plasmid that can be in connection with a whole cell conversion reaction (see Example 4).

Figure 9: Optimization of Cell Lysis by Ultrasound: Crude preparations of phenyl alanine dehydrogenase and malate dehydrogenase were prepared by ultrasound (see description in Example 6). Cells were exposed to ultrasound treatment for either 30 or 60 seconds and then cooled for 30 seconds, This treatment was repeated 2-8 times and the amount of pheDH and malate DH activity in the lysate was determined. Results are shown in the figure.

Figure 10: Optimum pH of Malate Dehydrogenase and PheDH Reactions. The effect of pH on the activity of pheDH and malate DH was examined and results are shown in the figure. The measurements were carried out with partly purified enzyme and are described in Example 7.

Figure 11: Optimum pH of Malate Dehydrogenase and PheDH Reactions. The effect of temperature on enzyme activity was measured and results are shown in the figure. The experiment is described in Example 7.

Figure 12: Formation Kinetics for Phenylalanine: A coupled reaction of pheDH and malate DH was carried out as described in Example 7. The formation of phenylalanine was monitored by HPLC over several hours and results are shown in the figure.

Examples

Example 1: Purification and biochemical properties of the MDH from E. coli

A Purification

The purification of the recMDH from *E. coli* crude extracts (expression strain: *E. coli* derivative JM105) was carried out in accordance with the purification protocol of Stols *et al.*(*Appl Environ Microbiol 63*:2695-701 (1997)). The rec-bacteria cells were first broken down by disintegration with glass beads (breakdown buffer Tris/HCl 100 mM pH 7.5). Thereafter, a purification step by a Q-Sepharose was carried out. After the purification by means of Q-Sepharose, it was possible to determine a specific activity of the MDH of about 7.3 U/mg. Using chromatography on hydroxyapatite and phenylsepharose, it was possible to purify the MDH to homogeneity with a specific activity of 133 U/mg.

Tab. 3: Summary of the purification of the rec-MDH from *E. coli.*

Purification step	Activity (U)	Protein (mg)	Spec. act. (U/mg)	Enrichment (-fold)	Yield (%)
Crude extract	210	202	1.03		100
Q-Sepharose	184	25.2	7.3	7.1	88
Hydroxyapatite	68	1.6	42.5	41	32
Phenylsepharose	24	0.18	133	129	11

B. Biochemical characterization

- Km values

For L-malate a Km value of 0.29 mM was measured, and for the coenzyme NAD^+ a Km value of 0.14 mM. Both Km values lie in a low range of <1 mM, and suggesting that

the two substrates are recognized by the enzyme with a good affinity. The two values suggest that the MDH can be used for the regeneration of NADH.

- Optimum pH (fig. 1)

MDH shows a maximum activity at relatively high pH values of 11 and higher. Nevertheless, the drop in activity at lower pH values is relatively small, thus 72% activity is still present at pH 8.0, and 67% activity is still present at pH 7.0.

- Optimum temperature

The optimum temperature of the MDH reaction is approx. 55°C (fig. 4).

Example 2: Coupling Reactions

A. Coupling of leucine dehydrogenase with malate dehydrogenase

The MDH from E. coli is coupled with an NAD-dependent leucine dehydrogenase (LeuDH from *Bacillus cereus*; Sigma). LeuDH catalyses the reductive amination of aliphatic keto acids, such as, for example, ketoisocaproate, to the corresponding L-amino acids, such as L-leucine, with consumption of NADH.

Test batch (1 ml in total; unless stated otherwise the concentration of the stock solution is stated in parentheses): 526 µl Hepes buffer (200 mM Hepes, pH 8.5 with 10 mM $MgCl_2$); 143 µl ammonium sulfate solution (500 mM in the test); 100 µl ketoisocaproate (100 mM); 20 µl NAD^+ (50 mM); 200 µl L-malate (500 mM, Na salt, dissolved in Hepes buffer, pH 8.5); 1 µl LeuDH (0.5 U in the test); 10 µl MDH (1.5 U in the test; partly purified). The test batch is incubated at 30°C, and after 0, 10, 30, 60 and 120 min samples are taken (50 µl; Eppendorf reaction vessels) and heated for 3 min at 95°C to stop the reaction. Denatured protein is separated off by centrifugation for 10 min at 13,000 rpm (Eppendorf bench centrifuge) and the supernatant is analysed by means of HPLC, after derivatization with ortho-phthalaldehyde (OPA).

Derivatization with OPA (= ortho-phthaldialdehyde):

140 µl Na borate buffer (100 mM; pH 10.4); 40 µl sample or standard; 20 µl OPA/IBLC reagent (= ortho-phthaldialdehyde/N-isobutyryl-L-cysteine). 20 µl of this reaction solution are injected for the HPLC analysis.

HPLC analysis: The results of HPLC analyses are shown in table 1.

B. Comparison experiment: Coupling of leucine dehydrogenase with formate dehydrogenase

In a comparison batch carried out in parallel, the same components as described above were used, but instead of the malate dehydrogenase, 0.5 U formate dehydrogenase (FDH from *Candida boidinii;* Sigma) was employed and instead of 100 mM L-malate 100 mM formate was used as the regeneration substrate. Results are shown in table 1.

Example 3: Coupling of the MDH with alcohol dehydrogenase:

The MDH from *E. coli* (expression strain: *E. coli* derivative JM 105) is coupled with an NAD-dependent S-specific alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH). The usability of the MDH is tested via the reduction of a ketone (p-Cl-acetophenone).

Test batch (1 ml in total; unless stated otherwise the concentration of the stock solution is stated in parentheses): 678.7 µl Hepes buffer (100 mM Hepes, pH 8.5 with 10 mM $MgCl_2$); 1.3 µl p-Cl-acetophenone (10 mM in the test); 20 µl NAD^+ (50 mM); 200 µl L-malate (500 mM, Na salt, dissolved in Hepes buffer, pH 8.5); 15 µl RE-ADH (1 U in the test); 85 µl MDH (1 U in the test; partly purified).

The test batch is incubated at 30°C, and after 0, 10, 20, 30 and 60 min samples (50 µl) are taken, 100 µl ethyl acetate are added and the upper phase is analysed by means of gas chromatography for the formation of the alcohol p-Cl-phenylethanol. Results are shown in table 2.

Example 4: Construction -of an expression vector with heterologous expression

From the sequence of the amplified fragment, primers with integrated restriction cleavage sites and a codon for the ribosomal binding site were constructed. After amplification of the malate dehydrogenase from recombinant pUC18, the PCR fragment was cloned into the recombinant recPhe-pKK-223-3 expression vector after the PheDH sequence at the PstI and HindIII restriction cleavage sites (fig. 8 – Construction of the plasmid for a heterologous expression for L-Phe synthesis by means of whole cell conversion).

PCR:

5' forward: N'-malic-pst

5' CTGCAGAGCCCAGGGATGGATATTCAAAAA 3'

concentration 100 pmol/µl

5' reverse: C'-malic-Hin

5' AAGCTTTTAGATGGAGGTACGGCGGTAGTC 3'

concentration 100 pmol/µl

Table 4: PCR protocol for amplification of the malate dehydrogenase from the recombinant pUC18 plasmid. The concentrations of the template DNA were varied.

Template DANN recpUC18	N'-malic-pst prim 1	C'-malic-Hin prim 2	dNTP	Buffer	Taq polymerase	H_2O
50 ng/µl	1 µl	1 µl	2 µl	10 µl	1 µl	83 µl
25 ng/µl	1 µl	1 µl	2 µl	10 µl	1 µl	83 µl
10 ng/µl	1 µl	1 µl	2 µl	10 µl	1 µl	83 µl

One cycle consists of:

Denaturing step : 94°C

Annealing step: 59°C

Amplification step: 72°C

Cloning:

The new construct of the recombinant plasmid (fig. 5) was transformed into competent *E. coli* cells JM 105 or HB 101. The standard transformation was carried out in accordance with the protocol of Hanahan (Hanahan, *J Mol Biol 166*:557-580 (1983)). For this, 100-200 µl of competent *E. coli* cells were thawed on ice and 40 ng of DNA from the ligation batch were added. The plasmid cell suspension was cooled for 30 min on ice and then heated at 42°C for 90 sec and immediately cooled again on ice. After addition of 300 µl LB medium, the cells were incubated for about 45 min at 37°C for regeneration. 200 µl of this culture were then plated out on to an LB plate containing antibiotic and incubated overnight at 37°C.

With the cloning of the malate dehydrogenase 3' to PheDH at the PstI and HindIII cleavage site with its own ribosomal binding site, expression of the two enzymes could take place simultaneously.

Example 5: Coexpression of PheDH and malate dehydrogenase

Certain positive clones were selected for expression. An individual colony of a clone was transinoculated into 5 ml LB_{amp} medium, and after $OD_{580} = 0.6$ was reached, cells were induced with 1 mM IPTG. The induction was carried out overnight and the harvested cells were broken down with ultrasound.

The recombinant strain HB101 shows a malate dehydrogenase activity of 100 U/ml and likewise a PheDH activity of 130 U/ml. The activity of the two enzymes in the recombinant strain JM 105 is clearly higher and is at ~ 600 U/ml for malate dehydrogenase and ~ 1,200 U/ml for PheDH. The two recombinant strains were cultured in a 10 l fermenter and the activity of the two enzymes was determined. Results are shown in Table 5.

Table 5: Determination of the activity of the expressed enzymes in a 10 l fermenter with LB medium as batch fermentation.

	Activity of PheDH		Activity of malate dehydrogenase	
	(U/ml)	(U/mg)	(U/ml)	(U/mg)
E. coli HB101	400	33	220	22
E. coli JM105	1300	118	640	71

It can be seen from the expression data that the *E. coli* strain JM 105 shows a significantly better activity for both enzymes, and all further experiments were therefore carried out with this strain.

Example 6: Optimization of the activity

To be able to utilize the maximum activity of the crude extracts obtained, several parameters were investigated and varied. The two heterologously expressed enzymes show the best stability properties under different conditions. It was of interest to determine the optimum properties for both enzymes in the same system. The following experiments were therefore carried out.

A. Optimization of the breakdown buffer

1 g of JM_{105} cells was broken down in 0.1 M Tris or 0.1 M Kpi buffer with/without BSA (1.5 g/l) 30%. Lysis was carried out with ultrasound over 70 cont. cycles. In addition to the buffers, 1.5 g/l BSA were added for stabilization of the enzymes.

Table 6: Comparison of the activity as a function of the breakdown buffer

	0.1 M Tris		0.1 M Kpi	
	- BSA	+ BSA	- BSA	+ BSA
PheDH	520 U/ml	610 U/ml	730 U/ml	1100 U/ml
Malate dehydrogenase	430 U/ml	720 U/ml	320 U/ml	610 U/ml

The addition of BSA led to an increase in the activity in both cases. The breakdown buffer also influenced the activities. It was to be seen here that the suitable breakdown buffer was different for the individual enzymes. The Kpi buffer was more suitable for the PheDH than for the malate dehydrogenase, but since the decrease in activity of the malate dehydrogenase in the Kpi buffer was relatively low, the recombinant cells continued to be broken down in this buffer after the heterologous expression.

B. Duration of lysis to maximize stability

The duration of the breakdown was also investigated and the critical point for stability of the enzymes during this operation was determined. The optimum duration of breakdown can be obtained from these data (fig. 9 – Stability determination of the PheDH and the malate dehydrogenase after various lysis times by means of ultrasound). The cells were cooled intermediately for 30 seconds after a 60 and 30 second treatment.

The following suspension was used for this experiment:

1 g recombinant cells (JM105)
3 ml Kpi buffer 0.1 M

During longer treatment of the cells with ultrasound, the activity of PheDH decreases drastically, whereas the activity of the malate dehydrogenase is retained. The ideal breakdown conditions for a 25% breakdown of 1 g of recombinant *E. coli* JM105 are therefore 4 x 30 s ultrasound treatment with 3 x 30 s intermediate cooling in an ice-bath. During longer treatments with ultrasound, the sample becomes heated, which can lead to denaturing of the enzymes. The amount of protein was determined in this experiment and can be seen in the following.

Table 7: Protein determination of the two enzymes expressed, PheDH and malate dehydrogenase, after variation of the breakdown time. The cells were cooled intermediately for 30 seconds after a 60 and 30 second treatment.

	60 sec x 2 spec. activity: U/mg	30 sec x 4 spec. activity: U/mg	30 sec x 8 spec. activity: U/mg
PheDH	30	105	16
Malate dehydrogenase	20	90	110

With a purification of the malate dehydrogenase to homogeneity, it was possible to achieve a specific activity of 466 U/mg. The purification steps are summarized in table 8.

Table 8: Purification of the recombinant malate dehydrogenase

	Volume (ml)	Activity (U)	Protein (mg)	Spec. activity (U/mg)	Yield (%)	Factor
Ultracen-trifugation	0.6	820	13	63	100	1
Hydroxy-apatite	9	470	5	94	57	1.49
Q-Sepharose	7	496	2.1	236	60	3.74
Phenyl-sepharose	1.9	280	0.6	466	34	7.39

C. Km value determination

K_M values were determined for the substrate and the coenzyme of malate dehydrogenase. The K_M values were determined on homogeneous or partly purified rec-malate dehydrogenase samples.

L-Malate: 0.29 mM

NAD^+ 0.14 mM

Example 7: Coupled L-phenylalanine synthesis with regeneration of the coenzyme NADH

Important factors for a coupled reaction are the optimum pH; the heat stability of the two enzymes; and other factors such as, *e.g.*, the influence of various substrates on the enzymes. With respect to optimum pH, a kinetics study was conducted and the pH-dependency of the synthesis was determined. The increase in activity at an increasing pH can be seen from fig. 10. Although the two enzymes do not show the highest activity at a pH of 8.0, this pH was chosen for the synthesis for coenzyme stability reasons.

A second important factor for the coupled enzyme reaction is the temperature at which the two enzymes remain stable for a relatively long period of time. A further experiment was therefore carried out to determine the optimum. As can be seen from fig. 11, the optimum temperature of both enzymes is 50°C. At 30°C the activity measured is only 60%.

The malate dehydrogenase is stable at 45°C for a relatively long period of time. However, since the PheDH becomes unstable at this temperature, syntheses were carried out at 30°C, so that it was possible to ensure the stability of both enzymes and of the coenzyme over a relatively long period of time.

The two enzymes were tested with two different buffers in each case in a reaction batch. Results are shown in Table 9.

Table 9: Comparison of the PheDH and malate dehydrogenase activity in various reaction buffers. The activities are to be seen in per cent of the optimum.

	0.1 M HEPES buffer (pH 8.0)	0.1 M Tris buffer (pH 8.0)
PheDH	84 %	100 %
MDH	100 %	42 %

Since the activity of the PheDH in HEPES buffer does not decrease substantially, the coupled enzyme reaction was carried out in this buffer. With the determination of the buffer, pH and temperature values, it was possible to select suitable conditions and media for the synthesis of phenylalanine by a coupled enzyme reaction with regeneration of the cofactor (NADH). 30 mM phenyl pyruvate, 100 mM ammonium sulfate, 100 mM HEPES buffer, 70 mM L-malate, 2 mM NAD^+, 2 mM Mg^{2+}, 25 U PheDH (partly purified) and 30 U malate dehydrogenase were employed. The samples are analysed by means of HPLC. The synthesis was monitored for several hours. After 4 h approx. 50% of the substrate employed, phenyl pyruvate, was converted into L-phenylalanine (fig. 12 – Formation kinetics for L-Phe. The formation of L-Phe was carried out *in situ*).

Example 8: Whole cell conversion

The following medium was used as the standard batch for the whole cell conversion:

0.1 M	HEPES buffer (pH 8.0)
40 mM	phenyl pyruvate
0.1 M	L-malate
0.1 M	ammonium sulfate
2 mM	$MgCl_2$

The conversion was carried out at 30°C and with 1 g of recombinant *E. coli* cells. The phenylalanine formed was detected by means of HPLC (fig. 6). The formation of L-Phe by recombinant *E. coli* cells was monitored for 20 h and the yield was determined (fig. 7). No metabolization of the product formed, L-Phe, is detected after incubation for 20 h.

What is Claimed is:

1. Process for the preparation of enantiomerically enriched organic compounds in a coupled enzymatic reaction system, comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO_2, characterized in that the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.

2. Process according to claim 1, characterized in that the first enzymatic transformation proceeds using an alcohol dehydrogenase or amino acid dehydrogenase.

3. Process according to claim 2, characterized in that an ADH from *Lactobacillus kefir* or *Rhodococcus erythropolis* is used as the alcohol dehydrogenase and a leucine dehydrogenase or phenylalanine dehydrogenase is used as the amino acid dehydrogenase.

4. Process according to one or more of the preceding claims characterized in that a malate dehydrogenase from *E. coli*, in particular *E. coli* K12, is used.

5. Process according to one or more of the preceding claims characterized in that the reaction is carried out in an aqueous single- or multi-phase solvent mixture.

6. Process according to one or more of the preceding claims, characterized in that the temperature during the reaction is between 20 and 40°C.

7. Process according to one or more of the preceding claims, characterized in that the pH during the reaction is between 6 and 9.

8. Coupled enzymatic reaction system for the preparation of enantiomerically enriched organic compounds, comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a

second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO_2, characterized in that the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.

9. Whole cell catalyst comprising a cloned gene for a first enzyme for transformation of an organic substrate and a cloned gene for a malate dehydrogenase, this being capable of preparation of an enantiomerically enriched organic compound in a first enzymatic transformation, NAD(P)H being consumed, and of allowing the regeneration of the NAD(P)H to take place in a second enzymatic transformation by malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO_2, wherein the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.

10. Plasmids containing gene constructs in which the gene for a malate dehydrogenase and a gene for an enzyme for transformation of an organic substrate with consumption of NAD(P)H are present.

Abstract

The present invention relates to a process for the preparation of enantiomerically enriched organic compounds. In particular, the present invention relates to an enzymatic process, in which, in a coupled enzymatic reaction system, NAD(P)H is consumed by one enzyme for the preparation of the organic compound and the NAD(P)H is simultaneously regenerated by a second enzymatic reaction. A reaction system which operates according to the invention in this manner and a whole cell catalyst are also proposed.

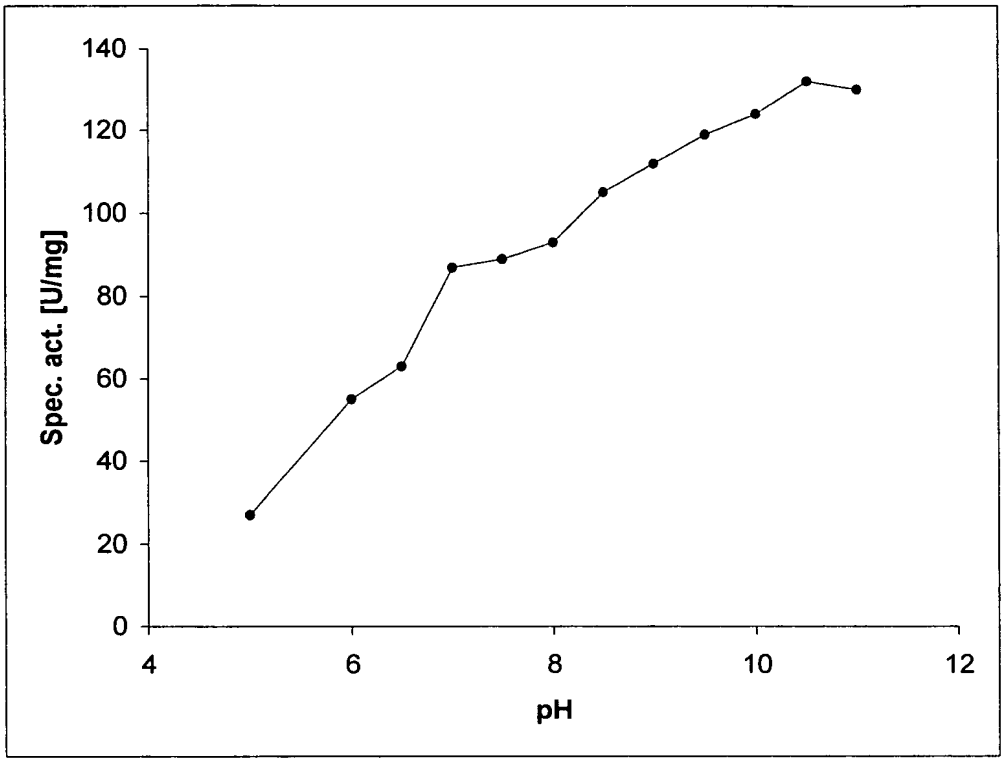


Figure 1

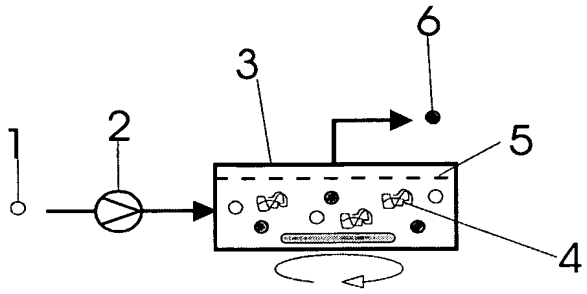


Figure 2

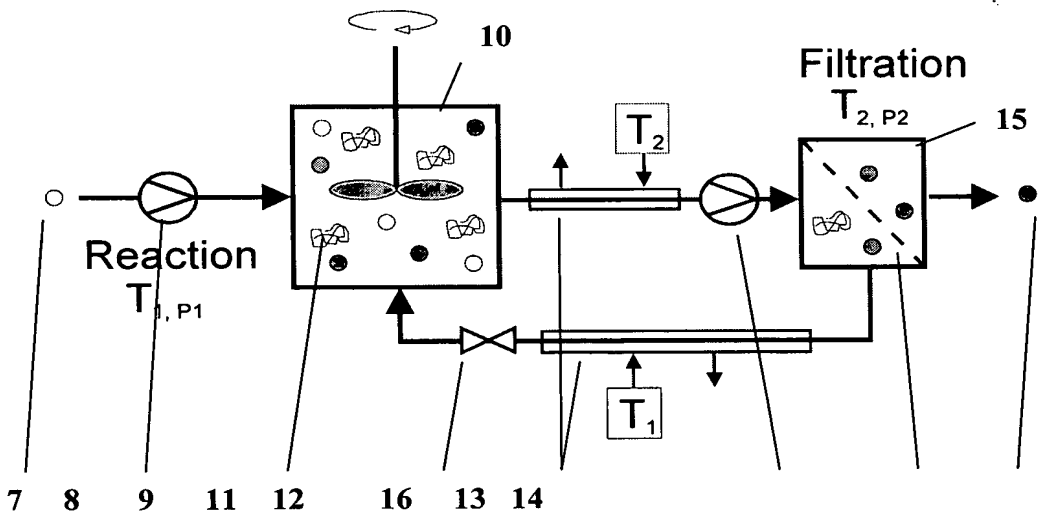


Figure 3

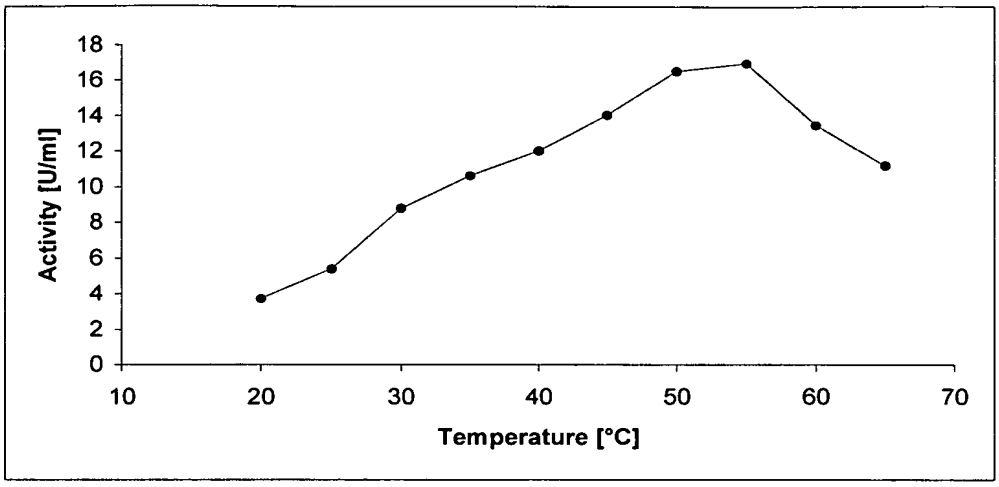


Figure 4

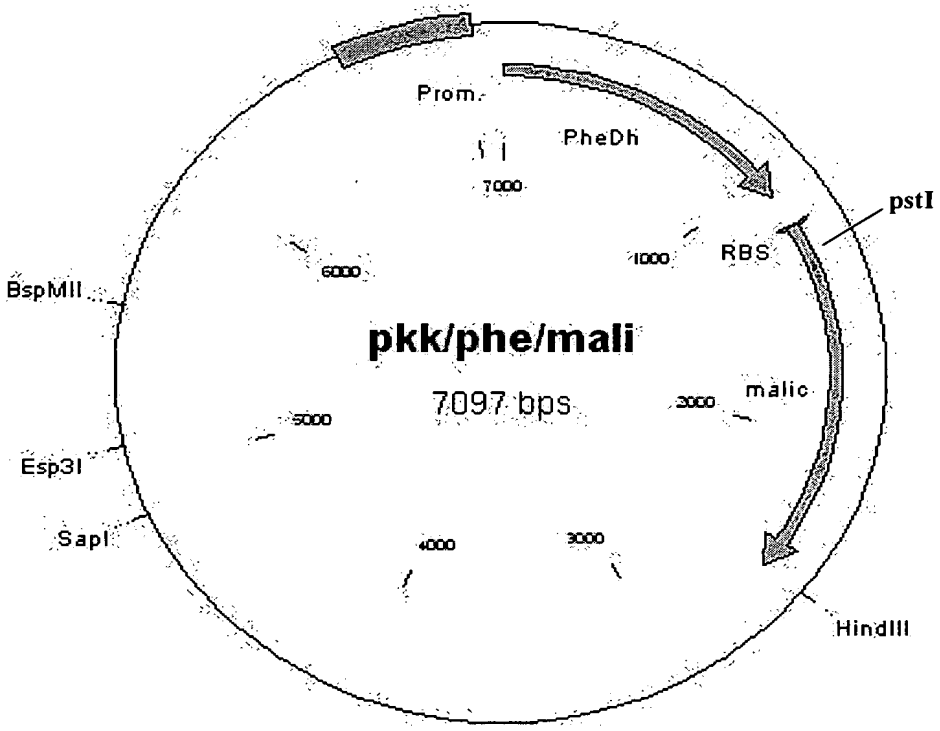


Figure 5

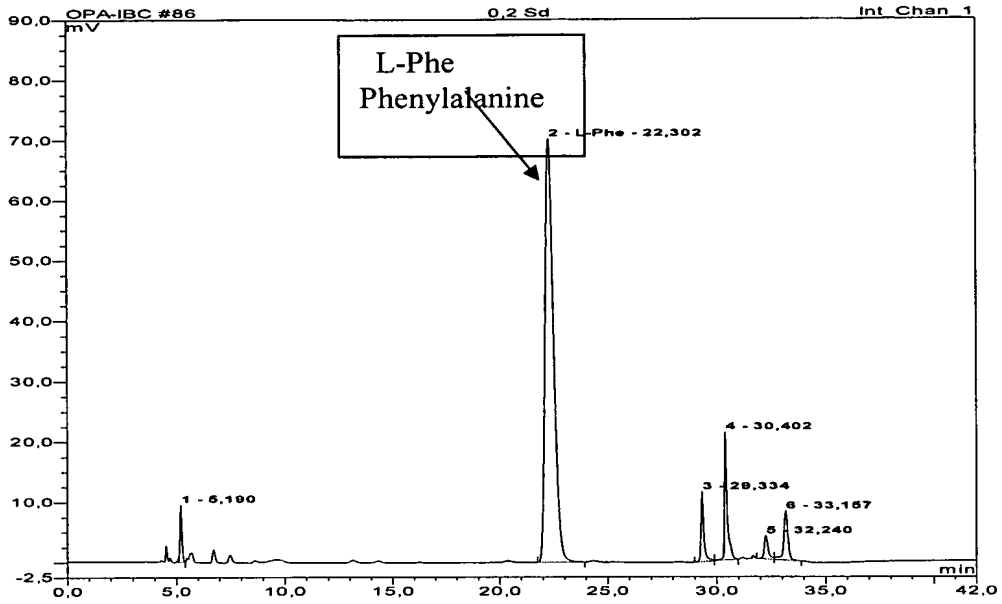


Figure 6

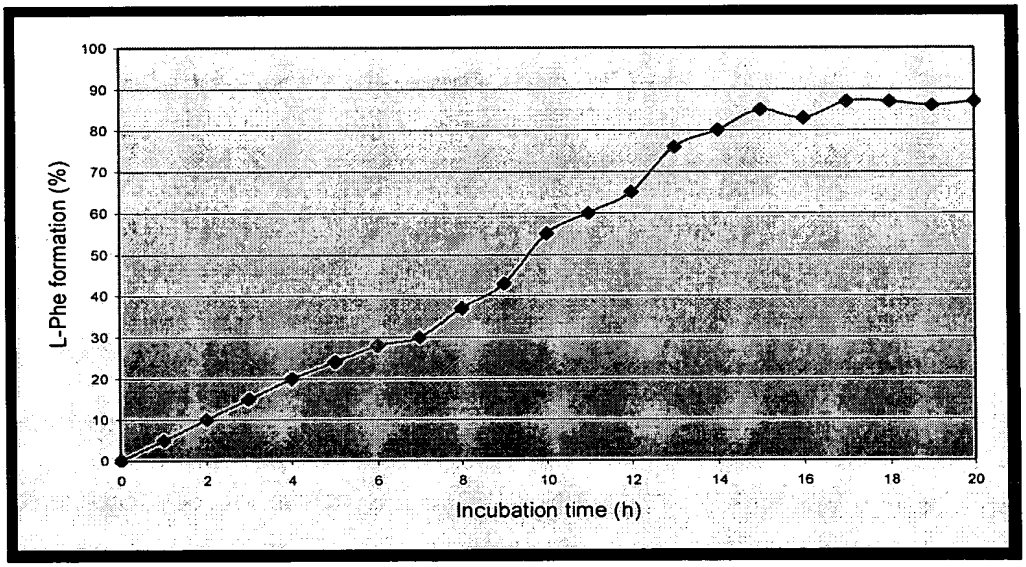


Figure 7

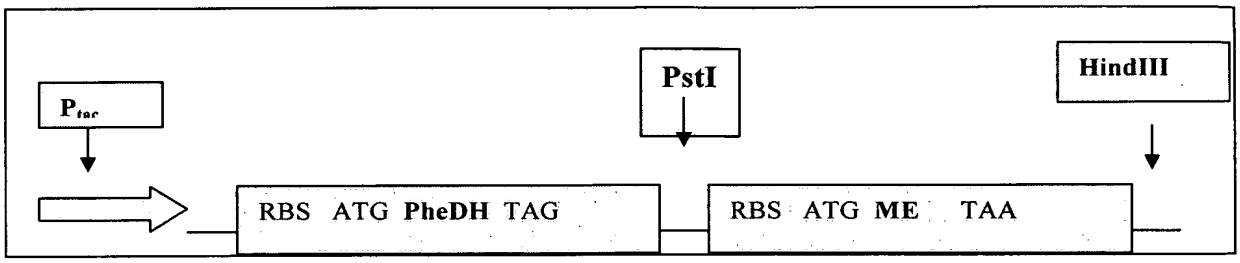


Figure 8

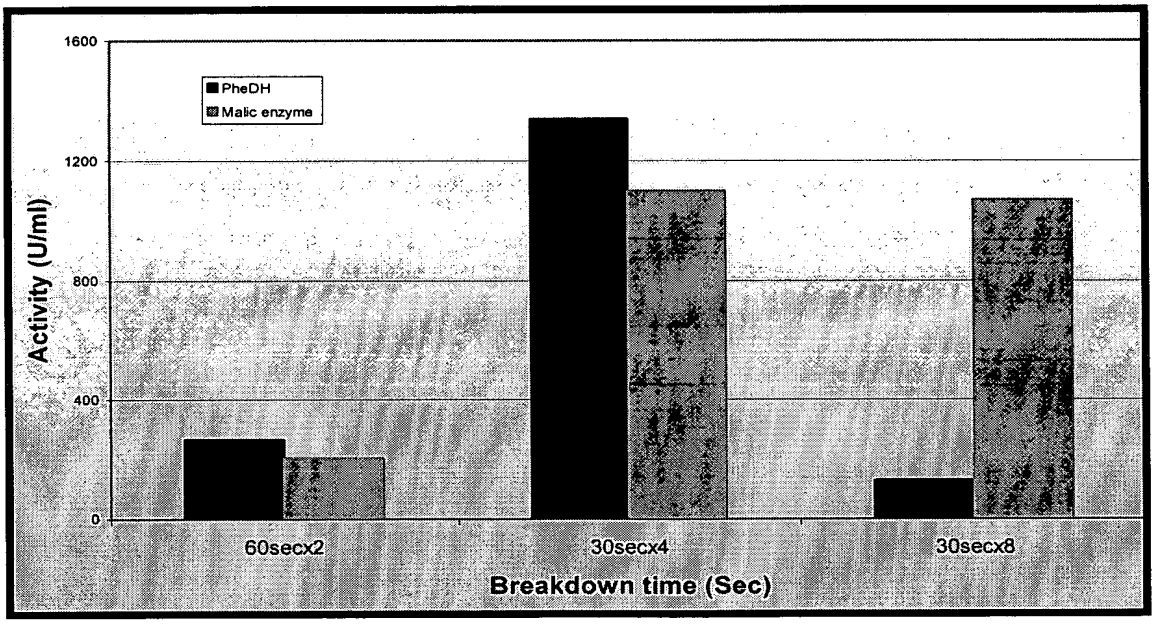


Figure 9

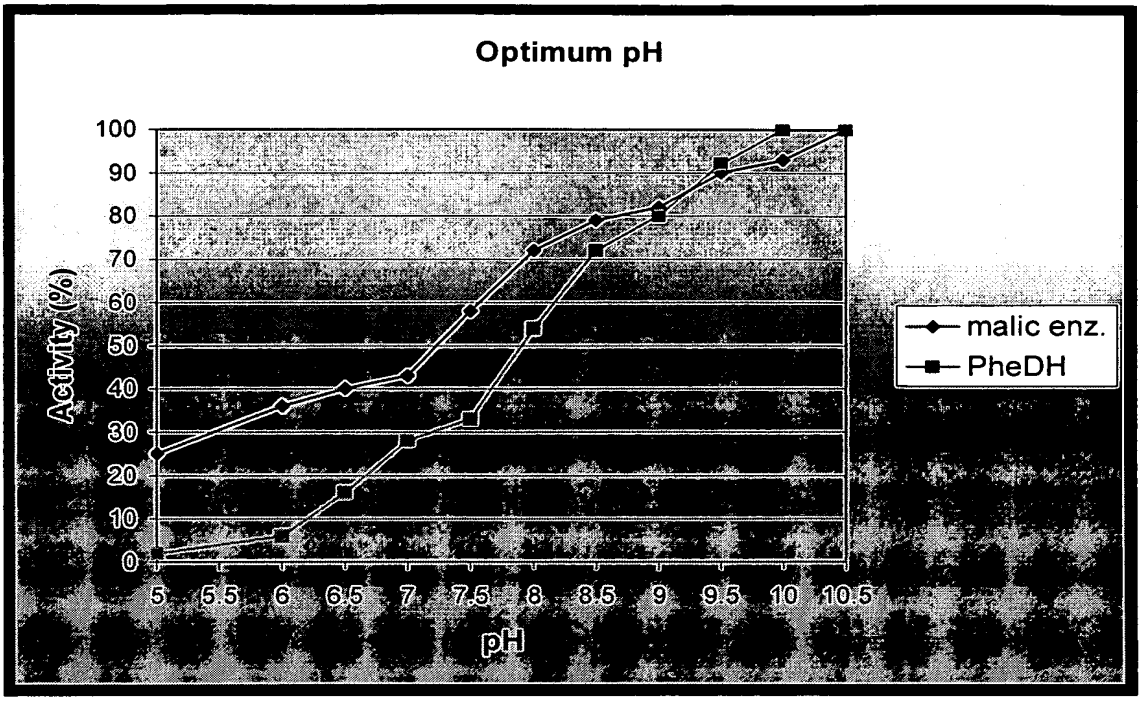


Figure 10

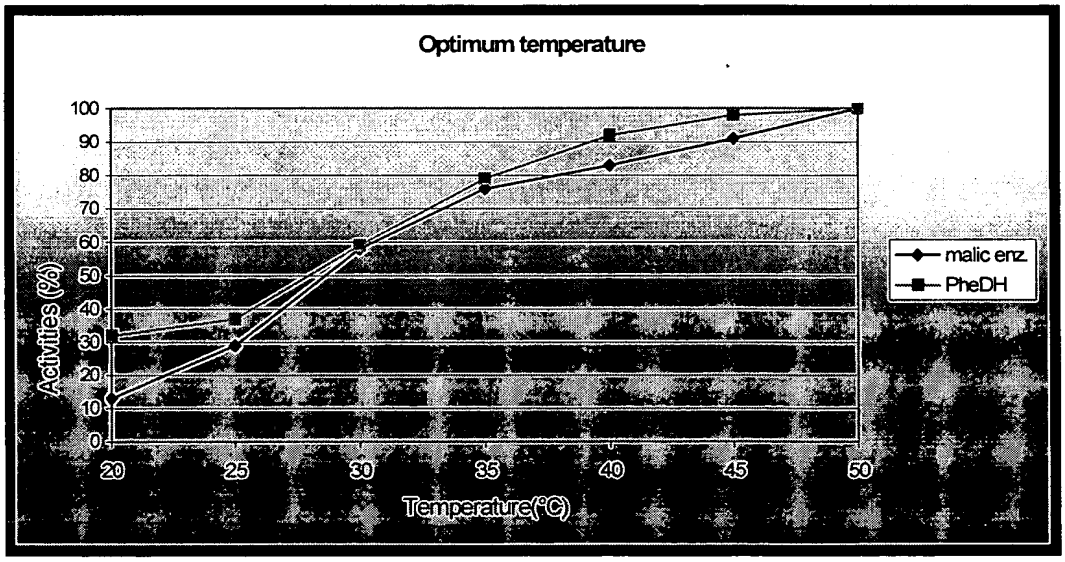


Figure 11

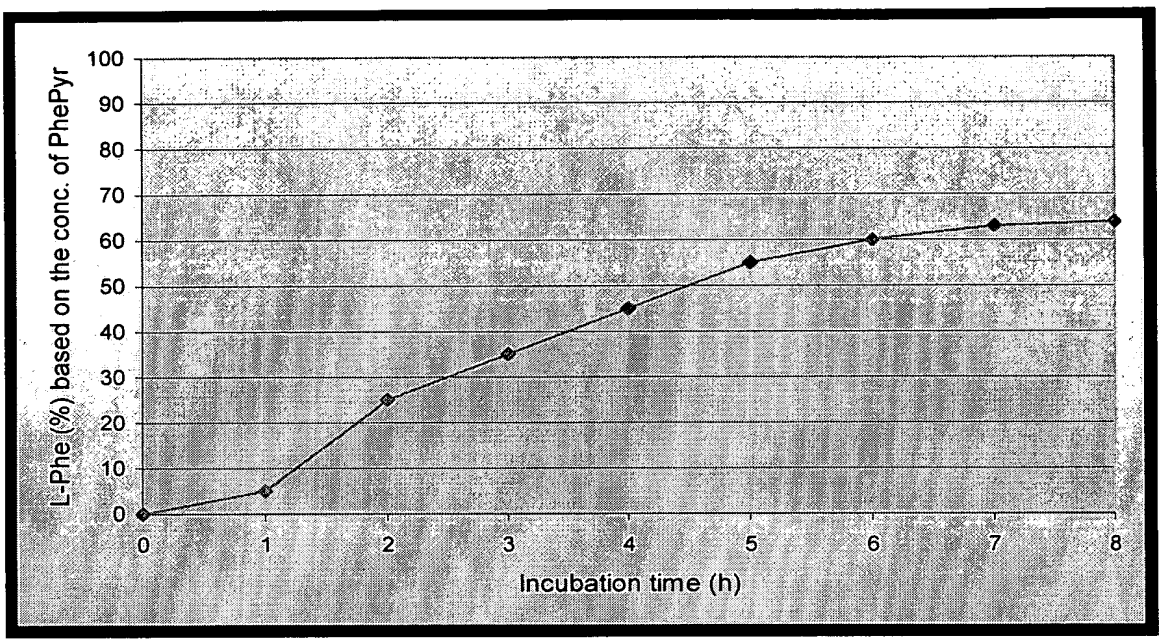

L-Phe (%) based on the conc. of PhePyr
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
Incubation time (h)

Figure 12